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13. ABSTRACT *(Maximum 200 Words)*

I had originally proposed to test the hypothesis that "biologically active derivatives of vitamin A (retinoids) inhibit mammary carcinoma cell proliferation by disrupting one or more growth factor activated serine/threonine protein kinase signaling cascades. Targets of these signaling cascades include genes that encode proteins required for progression through the cell cycle". The experimental approach was designed to identify the retinoid-regulated genes that negatively regulate cell cycle progression. Specifically, I proposed to identify and isolate genes whose expression is regulated by retinoic acid in hormone-dependent, but not hormone-independent cells, and determine if these genes encode proteins involved in cell cycle progression. To date we demonstrated that the alpha isoform of protein kinase C is retinoid induced in hormone dependent breast cancer cells and that the activity of this PKC is responsible for the retinoid-dependent disruption in mitogen signaling that results in growth arrest. The significance of this role of PKCα is underscored by our further finding that expression of PKCα in hormone-independent breast cancer cells allows retinoids to inhibit mitogenic signaling. In the long term, these results might open the door for developing combination therapy approaches that jointly target retinoid and PKC signaling.

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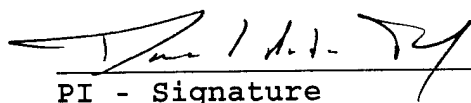
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INTRODUCTION

Breast cancer is the leading cause of female cancer deaths in this country and the incidence of new cases continues to rise. The reasons for this increase are unclear. Genetic cases account for only about 10% of breast cancers. A better understanding of other causative factors is required for developing alternative or additional therapies, and is required for the development and implementation of preventive interventions. Vitamin A status is a possible factor contributing to the development of many human cancers, including those of the breast. Epidemiological studies, experiments with animal models and clinical trials have implicated vitamin A status as an important factor in the development of a variety of human cancers (1-6). These studies establish a link between dietary retinoid intake and cancer risk, and further show that retinoids can prevent progression of some, but not all, human cancers. Despite these advances, a major question regarding the role of vitamin A and breast cancer remains unanswered. How, on a molecular level, do retinoids induce growth arrest of hormone-dependent breast cancer cells? I had originally proposed to test the hypothesis that **"biologically active derivatives of vitamin A (retinoids) inhibit mammary carcinoma cell proliferation by disrupting one or more growth factor activated serine/threonine protein kinase signaling cascades. Targets of these signaling cascades include genes that encode proteins required for progression through the cell cycle"**. The experimental approach was designed to identify the retinoid-regulated genes that negatively regulate cell cycle progression. Specifically, I proposed to identify and isolate genes whose expression is regulated by retinoic acid in hormone-dependent, but not hormone-independent cells, and determine if these genes encode proteins involved in cell cycle progression.

BODY

The initial proposal involved using a modified "enhancer trap" strategy to identify and molecularly clone RA-induced genes whose products were involved in negative regulation of cell cycle progression (7,8). As discussed in the annual report for year 1 of this project, difficulties were encountered in this approach. Specifically the cell line, T-47D in which we had characterized extensively the retinoic acid response, proved to be refractory to infection by retroviral based vectors. In last year's report I outlined several experiments that would be performed to (a) modify the infection protocol and (b) follow alternate avenues to identify and characterize RA induced target genes. Both of these approaches have been carried out and as a result, I am forced to abandon the enhancer trap strategy.

Fortunately we have generated strong preliminary data (actually fairly advanced, since 3 manuscripts are being prepared for submission within the next month) documenting the successful identification of several RA-induced genes, at least 2 of which mediate all or part of the anti-oncogenic action of RA in experimental systems. In the specific case of the T47D cell line, we have demonstrated an RA induction of the expression of protein kinase $C\alpha$ and protein phosphotyrosyl phosphatase 1C (PTP-1C)(9,10). We have now demonstrated that $PKC\alpha$ activity is required for the effect of RA on proliferation. In addition expression of $PKC\alpha$ in T47D cells in the absence of RA, mimics the effect of RA and finally we have demonstrated that expression of $PKC\alpha$ in the retinoid insensitive cell line MDA-MB-231 results in acquisition (or re-acquisition) of retinoid sensitivity (11). In addition, in a non-breast cancer based system, we identified an RA-induced cDNA, M61, that encodes a putative membrane bound peptidase, that when expressed in the absence of RA, inhibits de novo transformation by the viral oncogene middle T (12). During the coming (final) year of this project we will test the role of a PTP-1C in mediating the effect(s) of both RA and $PKC\alpha$ on T47D proliferation and on specific aspects of

signaling from the EGF receptor and from the heregulin receptors. We will also explore the possibility that the M61 peptidase is expressed in RA-treated and/or PKCα expressing T47D cells.

Progress on the original "Statement of Work":

Technical Objective 1: Insertional mutagenesis and molecular tagging of RARα–induced genes using an "enhancer trap" retroviral vector.
The original goal of Technical objective 1 was to develop and employ an "enhancer trap" based strategy for cloning RA-induced genes in the T47D breast cancer derived cell line. As described in detail last year we have had little success in infecting these cells with recombinant retroviruses. We had postulated that this might be the result of de novo methylation of the integrated viral vector DNA (13,14). We tested this prediction by infecting T47D and NIH3T3 cells in parallel with conditioned medium from a HEK293T-based packaging cell line transiently transfected with the pMV-7 retroviral vector (15). Total genomic DNA was isolated 24, 48 and 72 hrs post-infection, digested with Eco R1, resolved electrophoretically and then hybridized to ^{32}P-labeled vector DNA. No clear signal was seen in the T47D sample whereas a signal was readily apparent in the NIH3T3 sample. Therefore the defect was either at the level of infection (particle binding and uptake) or integration, and not at the level of proviral methylation. Since this presents a serious obstacle to the proposed studies, and since we have had very encouraging results using alternative approaches, the enhancer trap strategy is no longer being pursued. Despite our lack of success using the enhancer trap strategy in T47D cells, we believe that this approach could be viable in other systems, provided there is no inherent block to retroviral infection. We are anxious in the future to adapt it to other breast cancer cell lines such as the MCF7 line, or in cell lines from other human cancers that are retinoid responsive (e.g. from cervix, prostate and/or lung).

Task 1. Task completed in year 1.

Task 2. Task completed in year 2. Using transient transfection of cells with a CMV-GFP expression vector we were able to perform FACS analysis on live GFP-expressing cells in which the mean green fluorescence was 2 standard deviations above the mean for the entire population. The percent of the entire population within this reason approximated the percent seen by conventional fluorescence microscopy. This population was successfully collected and replated with high viability.

Task 3. This task has not been completed. See last year's report for alternative approach.

Task 4. This task has not been completed.

Task 5. This task can not be completed as originally proposed. See below for alternative approaches that are being pursued.

Technical Objective 2: Clone RARα-induced growth suppressing cDNAs.
Work being carried out in the laboratory by Ms. Ann Tighe (now Dr. Tighe) and Dr. Yunhi Cho (funded by DAMD pre- and post-doctoral fellowships, respectively) identified two proteins whose expression was induced following RA treatment of T47D cells. The first is the alpha

isoform of protein kinase C (9). The second is an SH2 domain containing, non-receptor tyrosine phosphatase known as PTP-1C (10). We have constitutively expressed PKCα in T47D cells. In our initial study we found that PKCα expressing cells grew slowly, did not respond to further treatment with retinoic acid, and that inhibition of PKCα activity, either in RA treated T47D cells, or in T47D-PKCα cells, significantly increased proliferation. Based on these results, and because of the technical difficulties discussed under Technical Objective 1 progress, we have focused on the role of PKCα as a critical RA-induced target gene. In addition we have initiated studies aimed at determining if PTP-1C is also critical for the RA-induced phenotype. Consequently we have made the decision to not pursue a subtractive cDNA cloning strategy from Am580 treated T47D cells at this time.

Task 6. This task has not been initiated.

Task 7. We have identified 2 genes whose expression is up regulated following RA treatment of T47D cells. A similar response was not seen in retinoid resistant MDA-MB-231 cells. These 2 are protein kinase Cα and the SH2 domain containing tyrosine phosphatase PTP-1C. In addition, we used an entirely different system to identify 8 other genes that are induced by RA (12), several of which have the properties predicted of RA-induced genes involved in growth regulation (these were summarized in Table 1 of last year's report).

Technical Objective 3: Characterization of function of RARα-regulated genes.

Task 8. We have constructed anti-sense expression vectors encoding PKCα and the putative peptidase M61. Analysis of the effect of expressing each of these in T47D cells will be completed during the coming year. In addition we have synthesized anti-sense oligonucleotides to inhibit expression of PTP-1C. Treatment of RA-induced T47D cells with these oligonucleotides is predicted to reverse growth arrest. This experiment is in progress.

Construction of cell lines expressing anti-sense RNAs for RA-induced genes.
PKCα. We have constructed anti-sense expression vectors for PKCα. Several vectors have been constructed including ones using transcriptional regulatory sequences from the MLV LTR, the RSV LTR and the rat β-actin promoter. In addition we have constructed an inducible anti-sense vector in which the RSV promoter is subject to negative regulation by the lacI repressor (expressed in trans). Isolation of T47D cell lines expressing these constructs is in progress.

PTP-1C. Because of the difficulties in establishing stably transfected T47D cells that I have documented above, we have chosen to use on anti-sense oligonucleotide approach to examine the requirement for PTP-1C (and PKCα) expression in establishing the RA phenotype. The following oligonucleotides have been designed and synthesized for this purpose. Each oligonucleotide was chosen to span the first AUG codon in the corresponding mRNA (underlined). The oligos were tested to ensure that they contained minimal amounts of internal secondary structure at 37°C/pH 7.5/physiological salt concentrations. Finally the sequences of each oligonucleotide were used in a BLAST search of Genbank. In both cases the only significant homology detected was to the human or rodent counterparts of the PTP-1C or PKCα gene.

Oligo sequences:
Antisense PTP-1C: 5'-GGACT TACCC ACGGG ACAG<u>C A</u>TCTC-3"
PTP-1C mismatch (4/25):   5'-GcACT TAgCC AcGGG ACAcG ATCTC

Antisense PKCα: 5'-CGTTG CCCGG GAAAA CGTCA GC<u>CAT</u> GGTC-3'
PKCα mismatch (5/29):   5'-CcTTG gCCGc GAAAA CcTCA GCCAT cGTC-3'

Task 9.   This task has been completed for PKCα.   The results are described in two manuscripts in preparation that should be ready for submission on or around December 1, 1999. Copies of the submitted manuscripts will be forwarded at that time. Similar experiments for PTP-1C and M61 will begin after demonstration that PTP-1C anti-sense oligonucleotides affect RA-induced growth arrest, and if M61 is RA-induced in T47D cells.

Both T47D and MDA-MB-231 derived cell lines have been isolated that constitutively express a PKCα cDNA. Expression has been confirmed by immunoblot analysis and by enzymatic assays (ref. 9, Figure 1 and data not shown). As noted above, constitutive expression of PKCα in T47D cells slows proliferation (to an extent equivalent to treatment with 10^{-8} M RA)(9). Detailed studies on RA-induced growth arrest of T47D cells demonstrated that: (1) RA increased population doubling times from 36 to >100 hr, without decreasing cell viability; (2) RA treatment significantly reduced EGF activation of the EGF receptor without altering EGFR expression levels (Figure 2); (3) RA treatment attenuated EGF signaling to the nucleus via MAPK cascades (Figure 2 & 3). This was evident both in reduced activation of MAPK, JNK and p38 by EGF in RA treated cells, and in a 50-90% inhibition of EGF induced transcription of c-fos and c-myc in RA treated cells (10). The effect of RA on EGFR activation and MAPK activation was totally reversed by prior treatment of cells with an inhibitor of PKCα. In addition to reduced proliferation (resulting from increased population doubling times and not cell death) T47D-PKCα cells were largely refractory to stimulation with EGF at the level of EGFR activation and MAPK activation, and induction of c-fos transcription (Figure 4). Of note, T47D-PKCα cells also expressed elevated levels of PTP-1C (Figure 4C), indicating that PKCα activity also mediated the RA-induction of this target gene.

Constitutive expression of PKCα in MDA-MB-231 cells (Figure 1) did not alter proliferation (Figure 5) or fetal bovine signaling to the nucleus (Figure 6). Therefore the lack of retinoid responsiveness of this cell line is not simply the result of the failure of RA to induce PKCα expression. Surprisingly (and of great interest), when PKCα expressing MDA-MB-231 cells were treated with RA, there was a significant decrease in proliferation (Figure 5), colony forming efficiency (not shown; ref 11), and serum induction of c-fos expression and MAPK activation (Figures 6 & 7). The role of PKCα in these changes in the response to RA were confirmed by expressing PKCα under a promoter in which expression was reversibly induced with IPTG (11). In these cells, RA responsiveness appeared ~24 hrs after adding IPTG to the media and persisted until IPTG was removed. After an additional 24 hrs, both PKCα (detected by immunoblotting) and RA responses were absent. The basis for the PKCα-dependent response to RA is not known.

Task 10. Since the 2 genes of primary interest were actually identified using immunoblotting, it is not necessary to sequence the corresponding cDNAs. Therefore Task 10 is no longer relevant to the goals of this project.

Studies Proposed for the Final Funding Period.   Studies planned for the final year of this project involve original Tasks 8 and 9 and to a limited extent Task 7. Task 8 will be completed first using an anti-sense oligonucleotide approach. In the initial experiments T47D cells will be treated with RA (10^{-9} – 10^{-6} M) and with 10 μM of the indicated oligonucleotides (sequences given above). At 24 hr intervals, from 1 – 5 days, cell numbers will be determined. In subsequent experiments T47D-PKCα cells will also be treated. I propose to reduce both the

labor involved, and the cost of the oligonucleotides, by studying the effects of these treatments on 1000 T47D or T47D-PKCα cells plated in single wells of 96 well dishes. All treatments will be done in triplicate (and the experiment repeated in either duplicate or triplicate). Cell numbers will be determined using the non-radioactive, CellTiter 96 system from Promega Corp. Conditions have been established for quantifying the RA induced arrest of T47D cell proliferation. If anti-sense oligonucleotide treatment reverses the effect of RA-treatment control experiments will be done on larger cultures to confirm that the oligonucleotides specifically inhibit expression of the target protein (PKCα of PTP-1C). At this point we will either continue with anti-sense oligonucleotides or turn to stable, anti-sense RNA expressing cell lines to confirm the role of PKCα and/orPTP-1C at the specific signaling steps discussed above.

In additional studies we will determine if the RA-induced M61 peptidase is expressed in T47D cells (+/- RA), MDA-MB-231 cells (+/- RA), T47D-PKCα or MDA-PKCα cells. If M61 is expressed in an RA-dependent manner that correlates with RA-induced growth arrest, then experiments similar to those described in the progress report (Task 9) and in the proposed studies (Task 8), will be initiated in the coming year.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that T47D cells can not be efficiently infected with retroviruses
- Identified PKCα and PTP-1C proteins up-regulated in retinoid treated cells
- Demonstrated that PKCα mediates the anti-proliferative action of retinoids in T47D cells
- Demonstrated that PKCα cooperates with retinoids to limit the proliferation of the normally retinoid resistant cell line, MDA-MB-231
- Identified 8 cDNAs up-regulated by retinoids in non-breast cancer cells that might mediate the effects of retinoids in breast cancer cells

REPORTABLE OUTCOMES

Cell lines constitutively expressing PKCα (derived from both T47D and MDA-MB-231 parental lines) and inducibly expressing PKCα (MDA-MB-231 only) as well as appropriate vector controls. In addition we have established an MDA-MB-231 cell line that expresses the lacI gene product that will be useful as a host for additional inducible expression experiments.

CONCLUSIONS

To date we have drawn two important conclusions. The first is a negative one. This is that the cell line initially chosen as a host for the enhancer trap cloning strategy is unsuitable for these experiments. This is the result of an as yet poorly understood resistance to expression of retroviral vector encoded genes. The second conclusion is more positive and enlightening. This is the finding that the alpha isoform of protein kinase C is retinoid induced in hormone dependent breast cancer cells and that the activity of this PKC is responsible for the retinoid-dependent disruption in mitogen signaling that results in growth arrest. The significance of this role of PKCα is underscored by our further finding that expression of PKCα in hormone-independent breast cancer cells allows retinoids to inhibit mitogenic signaling. In the long term, these results might open the door for developing combination therapy approaches that jointly target retinoid and PKC signaling.

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APPENDICES

Figure Legends.

Figure 1. MDA-MB-231 cells were infected with recombinant retroviruses encoding G418 resistance (vector control) or PKCα and G418 resistance (PKCα-MDA-MB-231). Stable G418 resistant cell lines were isolated and protein kinase C expression was measured by immunoblotting. PKCα was only detected in cells infected with the PKCα encoding retrovirus (cell line 3). In addition, constitutively expressing PKCα did not alter the expression profile of other PKC isoforms.

Figure 2. Serum starved T47D human breast cancer cells were treated with 10^{-6} M retinoic acid for 24 hrs. Cells were then stimulated for 0 – 60 min with 20 ng/ml epidermal growth factor (EGF) and activation of the EGF receptor or MAPK were assayed. In A by EGFR phosphotyrosine content was determined by immunoblotting of immunoprecipitated EGFR. RA treatment reduced the extent of EGFR tyrosine phosphorylation but not steady state EGFR levels. In addition, RA reduced EGF dependent EGFR tyrosine kinase activity as determined in immunecomplex kinase assays (B). Co-treating the cells an inhibitor of conventional PKCs, Go6976, reversed this effect of RA. MAPK activation was monitored both by following the change in mobility (C, top panel) that occurs following activation, and by probing immunoblots with an antibody that specifically recognizes only the active form of this kinase (C bottom panel). Treatment of cells with RA inhibited EGF induction of MAPK and the RA effect was reversed by co-treatment with Go6976 (Go). PD: PD98059, an inhibitor of MAPK kinase. Therefore the ability of retinoic acid to inhibit EGF signaling requires the activity of one or more conventional PKC. Since PKCα is the only conventional PKC expressed and only after RA induction of PKCα transcription (Cho et al. 1997), it must be mediating the EGF inhibitory activity of RA.

Figure 3. The ability of RA to inhibit mitogenic signaling is not limited to EGF and results in decreased transcription of the c-fos immediate early response gene. This was demonstrated by northern blot analysis of total RNA isolated from T47D cells stimulated for 30 min with EGF (20 ng/ml), insulin (1 μg/ml) or 17β-estradiol (10^{-7} M, E2). RA pretreatment (10^{-6} M, 18 hrs) reduced the expression of c-fos by >50% in each case. Similar reductions in c-jun and c-myc mRNA levels were also seen (Tighe et al., submitted). L30 is a ribosomal protein encoding cDNA that is used to confirm equal loading, transfer and integrity of the RNA samples.

Figure 4. Constitutive expression of PKCα in T47D cells inhibits EGF signaling. EGF induction of EGFR tyrosine phosphorylation (A) and MAPK activity (B) were compared in parental T47D cells and T47D-PKCα cells (see Cho et al., 1997) as described in Figure 2. Expression of PKCα reduced both measures of EGF signaling confirming the conclusions drawn from the results in Figure 2. In panel C we demonstrate that both RA treatment and PKCα expression increase the steady state level of the phosphotyrosine phosphatase, PTP-1C making this a candidate for limiting signaling from the EGFR to the cytoplasm.

Figure 5. The growth rate of MDA-MB-231 cells (filled circle) and PKCα expressing MDA-MB-231 cells (open circles) was compared in the absence (-RA) or presence of 10^{-6} M retinoic acid (+RA). Neither PKCα expression nor RA treatment affected MDA-MB-231 proliferation. However, RA treated PKCα expressing cells grew at a reduced rate.

Figure 6. The combined anti-proliferative effect of PKCα expression and RA treatment is associated with reduced serum induced c-fos expression. This was demonstrated by northern

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blot analysis of serum starved (0.5% FBS) or serum stimulated (10% FBS, stimulation was for 30 min, $\pm$ 10^{-6} M RA) parental MDA-MB-231 cells (cell line 1), vector control cells (line 2) or PKCα expressing MDA-MB-231 cells (line 3). Only in the PKCα expressing cells did RA inhibit the serum induction of c-fos. In contrast c-jun expression was elevated in PKCα expressing cells under all conditions, and RA inhibited induction of junB in all 3 cell lines.

Figure 7. PKCα expression also markedly attenuated serum induced MAPK activity. Serum activation of MAPK kinase was determined by immunoblotting. Whole cell extracts of MDA-MB-231 cells (A) or PKCα-MDA-MB-231 cells (B) after 0 – 30 min stimulation with 10% fetal bovine serum were probed with antibodies specific for the activated form of MAPK (Phospho-MAPK). Filters were stripped and re-blotted with antibodies that recognize both active and inactive MAPK (Total MAPK). In parental MDA-MB-231 cells RA had no effect on the magnitude or the duration of serum induced MAKP activity. In contrast, in PKCα-MDA-MB-231 cells the response to serum was reduced and when cells were co-treated with RA, eliminated.
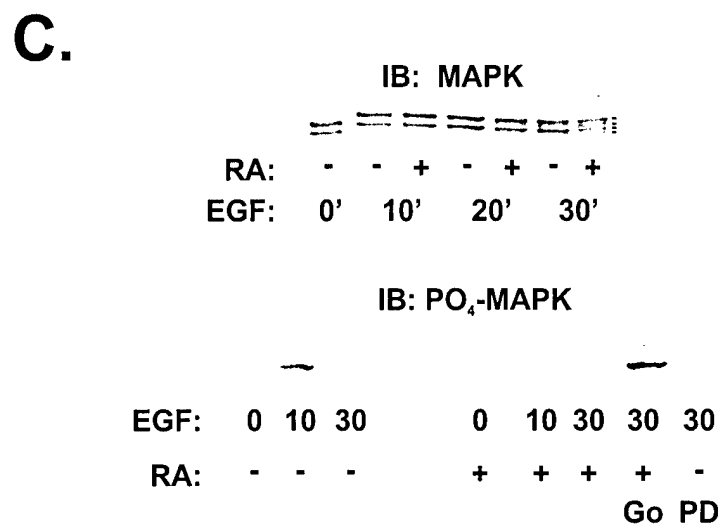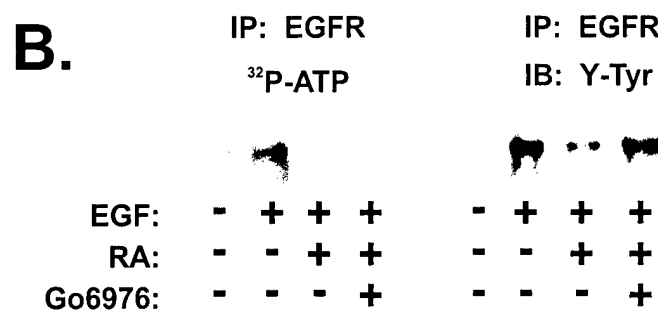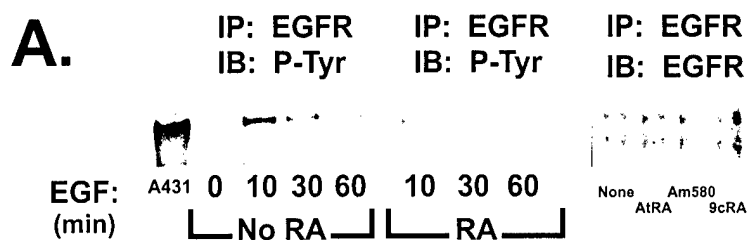
Figure 1. DAMD17-97-1-7289 Annual Report
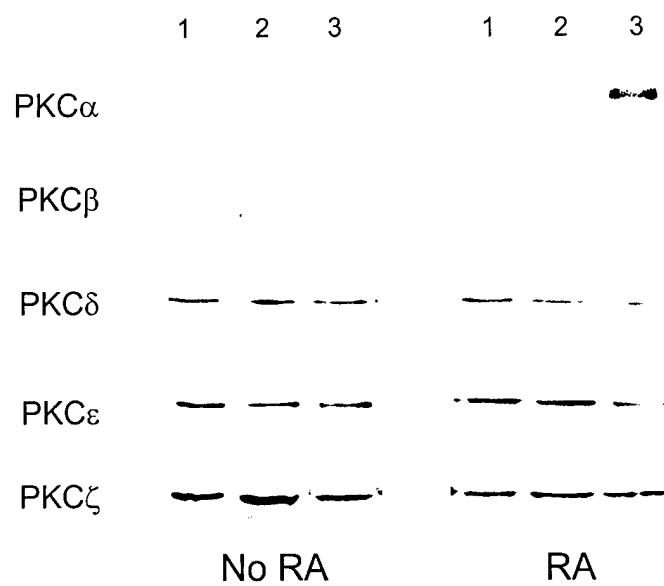
Cell lines:
1. Parental MDA-MB-231
2. Vector control
3. PKCα - MDA-MB-231

Figure 2. DAMD17-97-1-7289   Annual Report

A.

| IP: EGFR | IP: EGFR | IP: EGFR |
| IB: P-Tyr | IB: P-Tyr | IB: EGFR |

EGF: A431   0   10   30   60   10   30   60   None   Am580
(min)   └──No RA──┘ └──RA──┘   AtRA   9cRA

B.

IP: EGFR   IP: EGFR

^{32}P-ATP   IB: Y-Tyr

EGF:	-	+	+	+	-	+	+	+
RA:	-	-	+	+	-	-	+	+
Go6976:	-	-	-	+	-	-	-	+

C.

IB: MAPK

RA:	-	-	+	-	+	-	+
EGF:	0'		10'		20'		30'

IB: PO$_4$-MAPK

EGF:	0	10	30		0	10	30	30	30
RA:	-	-	-		+	+	+	+	-
								Go	PD

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Figure 3. DAMD17-97-1-7289   Annual Report

Stimulus: –   EGF   Insulin   E$_2$

RA: –   –   +   –   +   –   +

c-fos

L30

Figure 4. DAMD17-97-1-7289   Annual Report

A.

           T47D   T47D-PKCα

                                            IP: EGFR

EGF: 0' 10' 30' 60' 0' 10' 30' 60'   IB: P-Tyr

B.

          T47D   T47D-PKCα

                                      IB: MAPK

EGF: 0' 10' 30' 60' 0' 10' 30' 60'

          T47D   T47D-PKCα

                                    IB: PO$_4$-MAPK

EGF: 0' 10' 30' 60' 10' 30' 60' 0' 10' 30' 60'

C.

      T47D   T47D+RA T47D-PKCα

                              IB: PTP-1C

17

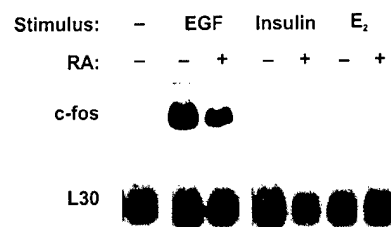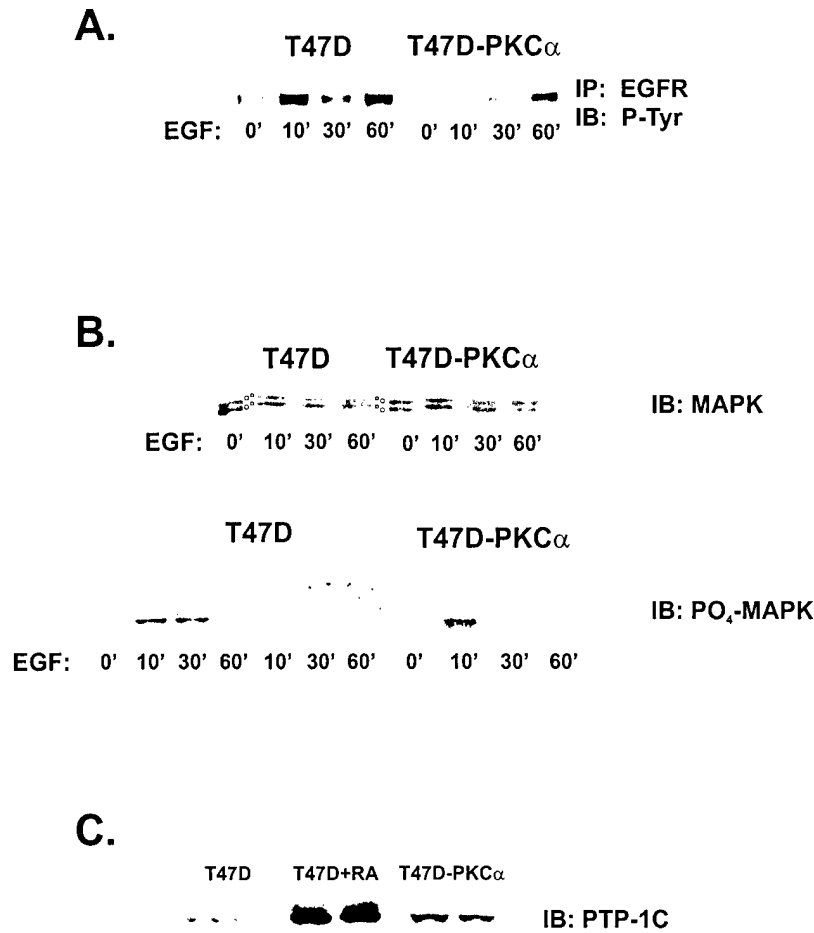# Figure 5. DAMD17-97-1-7289 Annual Report
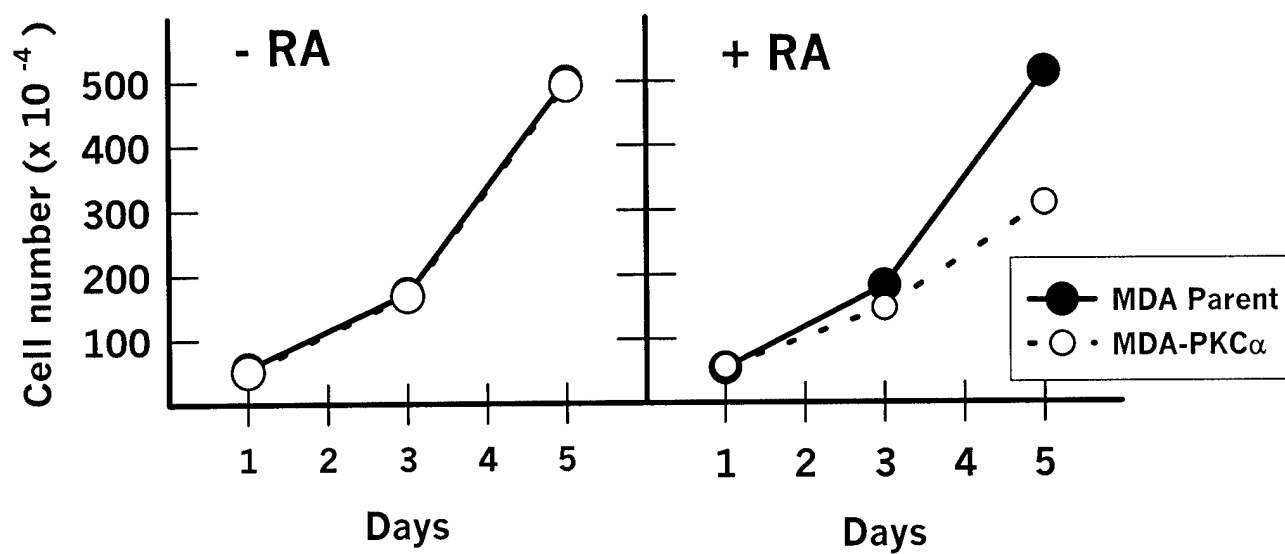
Figure 6. DAMD17-97-1-7289 Annual Report

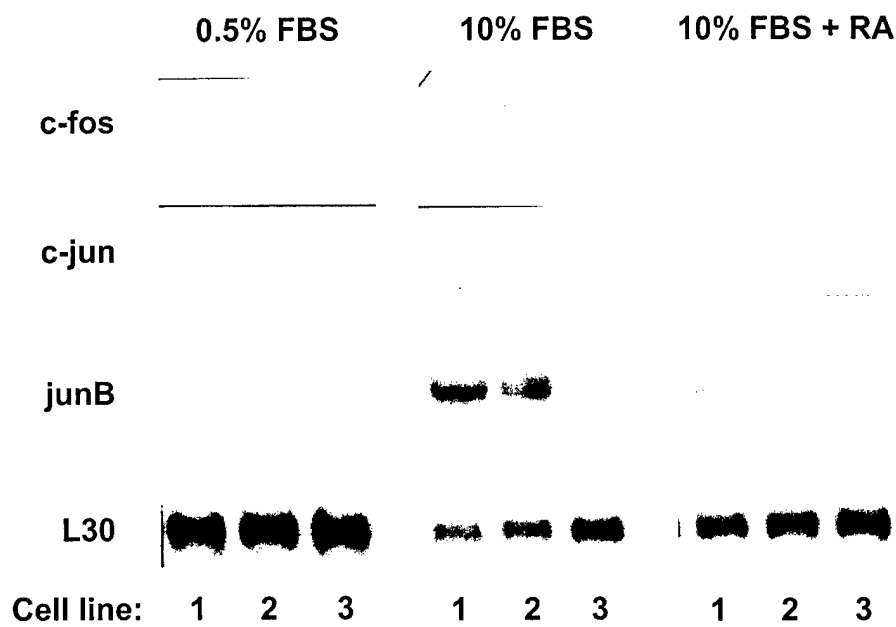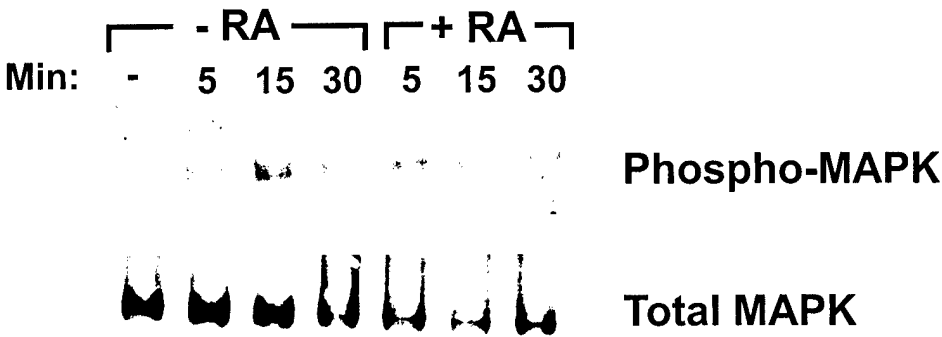|	0.5% FBS	10% FBS	10% FBS + RA
c-fos			
c-jun			
junB			
L30			
Cell line:	1 2 3	1 2 3	1 2 3

Cell lines:
1. Parental MDA-MB-231
2. Vector control
3. PKCα - MDA-MB-231

Figure 7. DAMD17-97-1-7289   Annual Report

A. MDA-MB-231

┌── - RA ──┐ ┌─+ RA ┐

Min: -   5 15 30   5 15 30

Phospho-MAPK

Total MAPK

B. PKCα–MDA-MB-231

┌── - RA ──┐ ┌─+ RA ┐

Min: -   5 15 30   5 15 30

Phospho-MAPK

Total MAPK